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Original Article

Age-specific prognostic significance of histone deacetylase 9 associated with TP53 according to immunohistochemistry in primary nasopharyngeal carcinoma: a survival analysis

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Abstract: Histone deacetylase 9 (HDAC9) was implicated in the progression of cancers, but showed conflicting functions in different cancers. To date, the function of HDAC9 in nasopharyngeal carcinoma (NPC) was still unclear. The purpose of our study was to assess the clinical significance and prognostic performance of HDAC9 in NPC. HDAC9 expression was specific reside within the nucleus of all NPC samples. Correlation analysis revealed that HDAC9 expression was only correlated with gender (r=0.324, *P*=0.003) in the subgroup of age ≤50 while showed inverse link with N stage (r=-0.327, *P*=0.007) in the subgroup of patients elder than 50. HDAC9 expression was associated with p53 expression (r=0.288; *P*=0.007) in the younger NPC patients (age ≤50), but showed no obvious relationship in the elder NPC patients (*P*>0.05). Although the prognosis of NPC patients with high HDAC9 expression showed no difference with those with low HDAC9 expression, HDAC9 expression performed quite opposite prognostic significance in two different age-cohort NPC patients. High HDAC9 expression independently predicted poor prognosis (overall survival, OS: 45.0% vs 67.2%, *P*=0.037; disease-free survival, DFS: 45.0% vs 67.2%, *P*=0.035) in the younger population (age ≤50); in contrast, HDAC9 expression showed no significant correlation with p53 expression and associated with quite good outcome in the elder population (age >50) (OS: 60.0% vs 32.6%, *P*=0.023; DFS: 55.0% vs 32.6%, *P*=0.023) though not independent (*P*>0.05). In conclusion, we firstly unveiled the different HDAC9 prognostic significance in different age-cohorts of NPC, and suggested HDAC9 expression to be a potential prognostic and therapeutic marker for this disease.

Keywords: Nasopharyngeal carcinoma, HDAC9, prognosis, tissue microarray, immunohistochemistry

Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy in southern China, the incidence which is up to 30 people/100000 people [1]. Despite the advance on the therapy depending on radiotherapy and chemotherapy which can improve the prognosis of NPC patients, the patients' 5-year survival rate remains low attributing to the high occurrence rate of recurrence or distant metastasis [2-4]. Thus study on the molecular markers associated with recurrence, distant metastasis or individualized treatment for NPC is under emergency to improve the clinical therapeutic effect and reduce mortality for this disease.

The acetylation of histone, as a reverse epigenetic modification regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), was important for normal and abnormal biological functions [5]. Dysregulated HDACs expression will perturb the balance of histone acetylation status, thus resulting in some diseases including cancer. For example, HDAC1/HDAC2 was recognized as a component of EZH2-HDAC1/HDAC2-Snail complex which could promote the NPC progression by repressing the E-cadherin transcription [6]. As a member of HDACs, HDAC9 regulates various biological functions, including cancers [7]. Many previous studies presented that HDAC9 played a pivotal but conflicting role in the progress of cancers

[8-13]. The aberrant expression of HDAC9 in cancer was first observed in glioblastoma, which was down-regulated [14]. Latterly, up-regulated HDAC9 expression was firstly observed in medulloblastoma and proved to act as an oncogene [15]. However, in lung cancer the expression of HDAC9 was down-regulated and was contributed to the suppressing of tumor progression [13]. To date, the HDAC9 function in NPC, especially the prognostic significance was not fully understood.

Herein, we plan to exam the HDAC9 expression status in NPC patients and tried to elevate the potential clinical significance, especially the prognostic significance of HDAC9, in order to provide more evidence on the molecular mechanism of HDAC9 expression in NPC.

Materials and methods

Clinical materials and ethnics statement

152 NPC patients were recruited from the People's Hospital of Guangxi Zhuang Autonomous Region for this study, which was approved by the Ethics Committee of the People's Hospital of Guangxi Zhuang Autonomous Region. All the patients were been first diagnosed as non-keratin NPC during the period from June 2006 to October 2008, and received no extra therapy before surgery. The follow-up was ended in January 2014, lasting 63-91 months. 71 patients died of NPC with a median survival time of 24 months; 32 patients were developing recurrence or metastasis; 77 patients were still alive, with a median survival time of 72 months (63 months-88 months); 4 patients were out of touch before the follow-up end time, and were excluded in the survival analysis.

Immunohistochemistry

All NPC specimens were fixed by formalin and sealed in paraffin, then the NPC tissue microarray (TMA) was made by Shanghai Outdo Biotech Co., Ltd. After deparaffinage by xylene and graded alcohol, TMA was incubated with EDTA to retrieve antigen and blocked with goat serum. TMA was incubated with the primary antibody anti-HDAC9 (1:80000, Abcam, ab-109446) at 4°C overnight. Lastly, sections were incubated with HRP (horse radish peroxidase) labeled antibody (DAKO, K8000) for 30 minutes, visualized by diaminobenzidine (DAB) system and hematoxylin re-dying. Three visual fields from different areas of each specimen were chosen at random. The positive rate scored as bellow: negative for 0, '1%-20%' for 1, '21%-40%' for 2, '41%-60%' for 3, '61%-80%' for 4, '81%-100%' 5. In each area, more than 100 cells were calculated for immunohistochemistry evaluation.

Statistical analysis

All statistical analyses were conducted using SPSS 17.0 software. The correlation between HDAC9 expression and clinical index was calculated by Spearman rank correlation coefficients and evaluated by Two-tailed Test. Kaplan-Meier method and log-rank test were used to analyze survival curves, and all potential predict markers were involved in COX multivariate regression survival analysis. $P<0.05$ was considered significantly.

Results

HDAC9 expressed in all NPC specimens and located in the nucleus

HDAC9 expression was evaluated by immunohistochemistry (IHC) staining of tissue arrays (TMA) including 150 World Health Organization type III NPC specimens and 2 World Health Organization type II NPC specimens. There are 112 male and 40 female whose age ranged from 18 to 77 years old with a median age of 48. The distribution of clinical stages were 5 cases of stage I, 57 cases of stage II, 45 cases of stage III, and 45 cases of stage IV. The detailed clinical data could be found in **Table 1**. The representative IHC images were shown in **Figure 1A-C**. In all NPC specimens, HDAC9 was specifically resided within the nucleus, while no HDAC9 expression was observed in the cytoplasm or membrane of NPC samples. HDAC9 expression was scored according to the positive rate, and the positive rate ≤85% was regarded as low expression, while >85% was divided into high expression.

Correlation between HDAC9 expression and clinical index according to the age of NPC patients

The correlation between HDAC9 expression and clinical index was evaluated by Spearman analysis. In the subgroup of age ≤50, HDAC9 expression was only correlated with gender ($r=0.324$, $P=0.003$). Nevertheless, HDAC9 ex-

Table 1. Correlation between HDAC9 expression and clinical index of the NPC patients in two different age-cohorts

Clinical factors	HDAC9 expression (Age ≤50)		Coefficient	*P* value	HDAC9 expression (Age >50)		Coefficient	*P* value
	Low	High			Low	High		
Gender			0.324	0.003			0.003	0.982
Male	46	8			39	17		
Female	15	12			7	3		
T stage			0.034	0.755			0.048	0.704
T1	5	1			3	2		
T2	25	8			21	6		
T3	16	5			8	6		
T4	15	6			14	6		
N stage			-0.099	0.378			-0.327	0.007
N0	17	7			12	11		
N1	32	11			21	8		
N2	10	1			10	1		
N3	2	1			3	0		
M stage			0.003	0.977			0.014	0.909
M0	59	20			44	19		
M1	2	0			2	1		
cTNM stage			0.054	0.630			-0.040	0.749
1	3	0			0	2		
2	22	8			18	6		
3	19	5			14	6		
4	17	7			14	6		
Recurrence or metastasis			0.003	0.979			-0.055	0.660
No	52	17			32	15		
Yes	9	3			14	5		

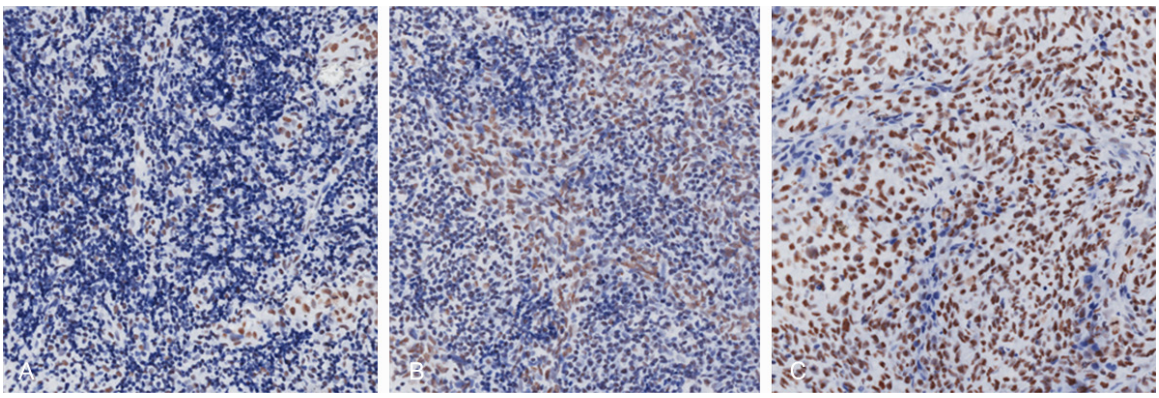

Figure 1. Immunohistochemistry of HDAC9: HDAC9 was specifically expressed in nucleus in nasopharyngeal carcinoma tissues. (A) low expression (B) median expression (C) high expression. (Magnification times: ×200).

pression was negatively correlated with N stage (r=-0.327, *P*=0.007) in the subgroup of age >50 (see **Table 1**). No significant link was observed between HDAC9 expression and tumor size, T stage, N stage, M stage and cTNM stage in both two age NPC population.

Table 2. Correlation between HDAC9 expression and TP53 expression

		HDAC9 expression	
		Age ≤50	Age >50
TP53 expression	Pearson Correlation	0.288	0.155
	P-value	0.007	0.215
	Number	85	66

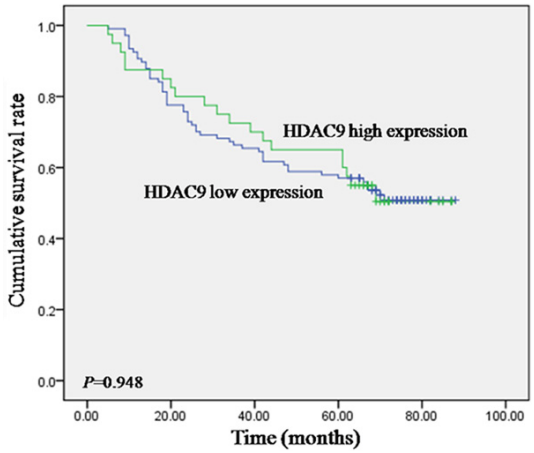

Figure 2. Survival analysis dependent on HDAC9 expression. *P* values were calculated by log-rank test.

Correlation between HDAC9 expression and TP53 expression in NPC

To better understand the potential regulatory mechanism of HDAC9 in NPC, we further analysis the association between HDAC9 and mutant TP53 expression in the two subgroups. The results shown as **Table 2** indicated that HDAC9 was positively correlated with TP53 expression in the subgroup of age ≤50 (r=0.288; *P*=0.007) but did not exist any statistical significance relation with TP53 expression in the subgroup of age >50 (*P*=0.215).

Age-specific correlation between HDAC9 expression and prognosis of patients with NPC

The expression of HDAC9 was not correlated with prognosis of NPC patients in this study (**Figure 2**). We further divided NPC patients into two different subgroups according to different age. In the subgroup of age ≤50, patients with high HDAC9 expression survive less time than those with low HDAC9 expression (45.0% vs 67.2%, *P*=0.037). In consistent, the DFS time of younger NPC patients (≤50) was inverse correlated with high HDAC9 expression level (45.0% vs 67.2%, *P*=0.035) (**Figure 3**). Conversely, high HDAC9 expression associated with good prognosis of NPC patients elder than 50 (OS: 60.0% vs 32.6%, *P*=0.023; DFS: 55.0% vs 32.6%, *P*=0.023).

Subsequently, the results of COX survival analysis showed that HDAC9 expression, clinical stage and recurrence or metastasis were all independent prognostic marker in the subgroup of age ≤50 NPC patients for both OS (*P*=0.021; *P*=0.004; *P*=0.000) and DFS (*P*=0.015; *P*=0.003; *P*=0.000). In the subgroup of elder NPC patients (age >50), no independent predict marker was detected in this study for OS, while HDAC9 expression, M stage, clinical stage and recurrence or metastasis were all independent predict markers for DFS (P=0.036; *P*=0.020; *P*=0.019; *P*=0.000) (**Table 3**).

Discussion

The function of HDAC9 in cancers was still under debate. The potential role of HDAC9 in NPC had not been fully understood. Here, our research studied on the HDAC9 expression and clinical significance, especially prognosis significance of NPC.

NPC was a common malignancy associated with EBV. However, the risk to develop NPC was depending on various factors, such as region, race, gender and age [16]. Among these factors, age has been validated as an important risk factor for NPC patients in China [17]. One previous report demonstrated that older NPC patients have advanced risk to develop NPC distant metastasis and had bad prognosis when compared with younger NPC patients [18]. According to these findings, one hypothesis that some factors involved in the progression of NPC might express in an age-specific pathway should be concluded. Some previous studies could partially support this assumption. For example, Tiwawech and colleagues demonstrated that p53 codon 72 polymorphism was presenting in an age-dependent way, and in different age groups, different p53 codon 72 polymorphism present different risk effects [19]. Based on these previous reports, we divided NPC patients into two subgroups according to different age, taking 50 for a split point since median age at natural menopause was 50 years in China [20].

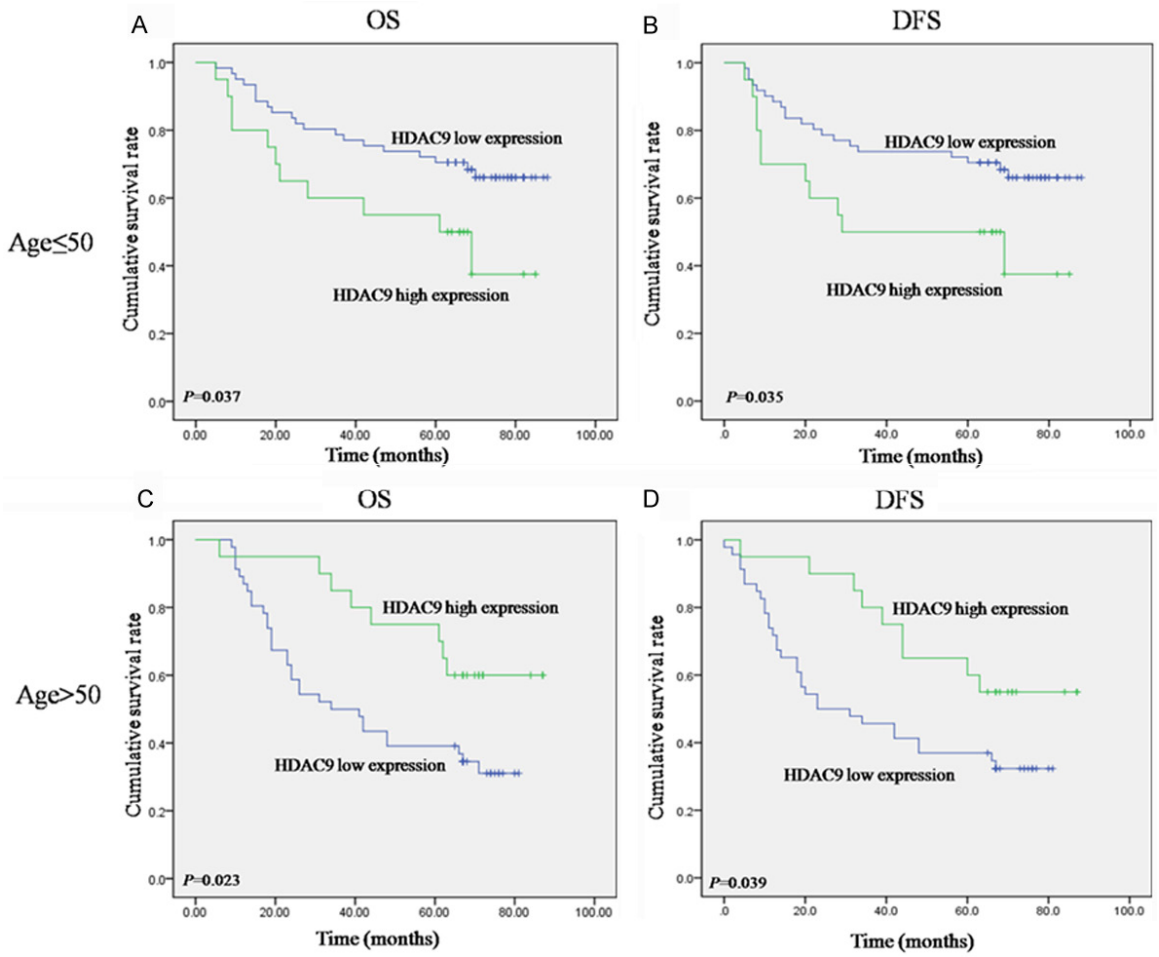

Figure 3. Survival analysis dependent on HDAC9 expression in different subgroup. High HDAC9 expression predict poor prognosis in the subgroup of NPC patients 50 years old or younger in (A) OS and (B) DFS; high HDAC9 expression predict good prognosis in the subgroup of NPC patients elder than 50 in (C) OS and (D) DFS. *P* values were calculated by log-rank test.

HDAC9 expression was specific reside within the nucleus of all NPC samples, in consistent with the previous studies which investigated the HDAC9 expression in various types of cancers and localized HDAC9 expression in the nucleus in various cancer and normal cells. The correlation between HDAC9 expression and clinical index showed different features in different age subgroups. HDAC9 expression was only correlated with gender in the subgroup of age ≤50 while showed intense inverse link with N stage in the subgroup of patients elder than 50. Survival analysis indicated very opposite relationship between HDAC9 expression and overall survival time. High HDAC9 expression associated with p53 expression, and independently predicted poor prognosis in the younger population (age ≤50); in contrast, HDAC9 expression showed no significant correlation with p53 expression and associated with quite good outcome in the elder population (age >50) although not independent ($P>0.05$). Based on these findings, we speculated that HDAC9 implicated in the progression of NPC by an age-specific pathway. HDAC9 might act as an oncogene by impeding the apoptosis of tumor cells according to improve the inactive mutation p53 expression in the younger NPC patients; in the subgroup of patients elder than 50, the master function of HDAC9 might be inhibition the metastasis ability of tumor cells, thus prolong the overall survival time of patients.

Further analysis unveiled that HDAC9 expression showed different clinical significance in the two age cohorts. In the age ≤50 population, high HDAC9 expression associated with poor prognosis, and positively correlated with

Table 3. Cox regression analysis under inclusion of clinical factors and HDAC9 expression in NPC

	Age ≤50					
	OS			DFS		
	P-value	Exp (B)	95.0% CI	*P*-value	Exp (B)	95.0% CI
HDAC9 expression	0.021	2.483	1.147-5.375	0.015	2.595	1.205-5.587
T stage	0.147	0.541	0.235-1.242	0.086	0.498	0.225-1.104
M stage	0.273	2.452	0.493-12.179	0.604	1.520	0.311-7.423
cTNM stage	0.004	4.446	1.596-12.383	0.003	4.130	1.645-10.373
Recurrence and metastasis	0.000	4.723	2.112-10.562	0.000	7.666	3.349-17.552
	Age >50					
	OS			DFS		
	P-value	Exp (B)	95.0% CI	*P*-value	Exp (B)	95.0% CI
HDAC9 expression	0.180	0.548	0.228-1.321	0.036	0.409	0.177-0.944
Gender	0.290	0.441	0.097-2.007	0.260	0.426	0.096-1.884
T stage	0.387	0.678	0.281-1.636	0.077	0.470	0.203-1.086
N stage	0.197	1.339	0.859-2.087	0.933	0.982	0.639-1.508
M stage	0.085	3.376	0.844-13.504	0.020	6.497	1.342-31.443
cTNM stage	0.153	2.306	0.734-7.246	0.019	3.702	1.240-11.056
Recurrence and metastasis	0.069	1.994	0.947-4.198	0.000	5.284	2.428-11.499

mutant p53 expression. Elevated p53 expression was observed in NPC and had been demonstrated to be functional in NPC cells. Wild p53 expression was proved to be functioned as a tumor suppressor by promoting tumor cell apoptosis. Mutant p53 expression was observed in cancers and was implicated in the oncogenic progress of cancers. The regulatory function of HDAC9 on p53 expression had also been demonstrated in osteosarcoma [10]. The intense linked between HDAC9 expression and mutant p53 expression provided a possible that HDAC9 might promote the progression of NPC by promoting the formation of mutant p53 and thus inhibit the apoptosis of tumor cells, which might illustrate the oncogenic function of HDAC9 in younger NPC patients.

On the other hand, HDAC9 acted as a tumor suppressor in elder NPC patients. The tumor suppress function of HDAC9 had been validated in lung cancer [13]. However, the regulatory mechanism of HDAC9 had not been fully understood. One previous finding that might support the tumor suppression function of HDAC9 was the inhibition function of HDAC9 acted on a tumor promoter ATDC (TRIM29) [21]. Considering the inverse link between HDAC9 expression and N stage unveiled in this study, the tumor suppression of HDAC9 expression acted in NPC possibly performed by inhibiting the regional metastasis. Further investigate focusing on the HDAC9 expression affection on NPC cells metastasis ability needed to be done to support our assumption.

In conclusion, we firstly unveiled the different HDAC9 prognostic significance in different age-cohorts of NPC, and suggested HDAC9 expression to be a potential prognostic and therapeutic marker for this disease. Moreover, we suggested age to one important factor should be considered when NPC patients received the treatment of HDAC9 inhibitors.

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Disclosure of conflict of interest

None.

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